Analyst

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Enhanced determination of As-phytochelatin complexes in *Chlorella vulgaris* using focused sonication for extraction of water-soluble species

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

The most challenging areas in the analysis of As-GS/PC complexes are their extraction from small amounts of biological material and the maintenance of their stability during HPLC separation. Focused sonication was used to extract these complexes from *Chlorella vulgaris* and the integrity of such complexes was determined by HPLC online with simultaneous HR-ICP-MS and ES-MS/MS detection. Water soluble arsenic species were extracted with an improved 71.1% (SE 0.78) efficiency and much reduced extraction times (30 s) allowing the determination of unstable arsenic phytochelatin (PC) and glutathione (GS) species in small biomass making the method particularly well-suited for cell cultures. Here, it was found that *C. vulgaris* produces the following intact phytochelatins and homo-phytochelatins (with Ala and desGly instead of Gly) complexes when cells are exposed to As(III): As(III)-PC_2, GS-As(III)-PC_2, As(III)-$(PC_2)_2$, MMA(III)-PC_2, As(III)-PC_3, As(III)-PC_4, As(III)-γ-$(Glu\text{-}Cys)_3$-Ala, GS-As(III)-γ-$(Glu\text{-}Cys)_2$-Ala, As(III)-γ-$((Glu\text{-}Cys)_2)_2$-Ala, MMA(III)-γ-$(Glu\text{-}Cys)_2$-Ala, As(III)-γ-$(Glu\text{-}Cys)_2$, GS-As(III)-γ-$(Glu\text{-}Cys)_2$. When the alga was exposed to DMA, only $DMAS^V$-GS was found. In contrast, cells did not produce any complex when exposed to As(V). It is the first time that, as a result of the newly developed extraction method using sonication, such complexes have been identified in *Chlorella vulgaris* exposed to arsenic and that intact arsenic homo-phytochelatins have been reported in any organism.

Introduction

Inorganic soluble arsenic is highly toxic and one response of plants upon exposure to arsenic is the production of glutathione (GSH or if bound to arsenic as GS), phytochelatins (PCs) and its homologues (where Gly is substituted by Ala, Ser, Glu and Gln or is absent)[1,2] which form complexes with As(III) and to a lesser extent, As(V)[3,4]. However, the identification and quantification of As-GS/PC complexes has proven to be elusive due to their instability thus presenting a challenge for modern speciation techniques[5]. The most problematic steps in the analysis of these complexes are their extraction from the biological material and the maintenance of their stability during HPLC separation[6]. Although conditions for stability of the complexes in a mobile phase and HPLC column have been achieved[5,7,8], relatively little progress has been made in ensuring the integrity and representativeness of As-GS/PC complexes when extracting from solid samples or cells such as the green microalga *Chlorella vulgaris*. Murray et al.[9] studied the biotransformations of arsenate in *C. vulgaris*, and found that when extracting with 1:1 (v / v) methanol/water followed by 10 min in a sonication bath, the extraction efficiency was between 11% to 27%, a surprisingly low figure compared to other algae. This led to the conclusion that the arsenic species found were not representative of the species present in the algal cells. Another group investigated the blue green-alga *Nostoc commune var. flagelliforme* and obtained an extraction efficiency of 34% when using the same extraction conditions[10].

Several methods have been used in the attempt to achieve quantitative extraction from biological samples. These include extraction in water and various water/methanol ratios, assisted with combinations of bath sonication, accelerated solvent extraction as well as harsher acid microwave-assisted extraction[11]. However, these reported methods tend to focus on maximising the recovery of arsenic often at the expense of species stability.

Conditions for the stability of As-GS/PC during extraction and analysis rely upon:

i pH of the solution: acidic and compatible with both Inductively Coupled Plasma (ICP) and Electrospray Ionization (ES) i.e. 0.5%-1% formic acid[5];

ii choice of separation column: a strong anion exchange column can break As-S bonds; To date only a reversed phase C_{18} column has been found suitable to fulfil the required conditions[5,7];

iii temperature of the solution: it has been found that during freeze-drying more than 90% of As-PC_3 and other small peptides such as PC_2 and PC_3 can be lost and that temperatures above 4 ºC do not favour integrity[5] and;

iv time of analysis: integrity of the complexes is only

guaranteed for 4 h[5].

Currently, the only method used routinely to extract As-GS/PC complexes consists of extraction with 0.5-1% (v / v) formic acid for 1 h at 4 ºC. This method requires grinding the samples in liquid nitrogen, a process which may compromise the complex integrity and parts of the sample can be lost[3,5,12]. Extraction efficiencies of 87 ± 6% (n = 28), 77 ± 19% (n = 28)[13] and 92 ± 9% (n = 42)[14] have been reported in plants (roots, stems and leaves) and seaweed tissues.

In focused sonication, ultrasound energy is applied directly to create cavitation bubbles that can break and disrupt intermolecular interactions. It conveys 100 times more energy than an ultrasonic bath[7] and if used correctly can provide conditions conducive to complex stability while avoiding the necessity to freeze and grind the cells in liquid nitrogen. Extraction can be performed in 1% formic acid, at low temperature (4 ˚C, using an ice bath) with a fast extraction time (30 s). Salgado et al.[15] studied arsenic extraction efficiency from *C. vulgaris* cells using focused sonication and compared it to magnetic stirring and bath ultrasonication. Optimal extraction was achieved with 5 mL of deionised water per 200 mg of dry algae and 30 s of focused sonication. The best efficiency was found at 64 ± 3% using focused sonication for 30 s. The study included speciation of inorganic arsenic with regards to oxidation state and ligands but the stability of unstable As-GS/PC species was not investigated[15].

This study describes a technique to extract As-GS/PC complexes that offers considerable improvement on existing reported methods. Using this method, we report a number of As-GS/PC complexes that have not been previously observed in *C. vulgaris.* In addition, intact arsenic homo-phytochelatins are reported for the first time in any organism. This represents a novel procedure designed to extract As-GS/PC complexes in *C. vulgaris* cells and potentially small quantities of any non-fibrous organic matrix and will enable further understanding of mechanisms involved in detoxification by algal cells exposed to inorganic arsenic.

Materials and methods

All reagents, growth media and eluents were made with 18.2 MΩ cm deionised water from Purite (Thame, UK). Sodium arsenite (As(III)), gallium and arsenic solutions (ICP 1 g L^{-1} in 2% HNO_3) and nitric acid (trace metal analysis) were purchased from Fisher Scientific (Loughborough, UK). Sodium arsenate dibasic heptahydrate (As(V)), dimethylarsinic acid (DMA), HPLC grade formic acid and methanol were purchased from Sigma (Dorset, UK). All glassware was washed with alkaline laboratory detergent (Fisher Scientific), allowed to soak overnight in 10% HNO_3 solution, triple rinsed with deionised water and allowed to air dry.

C. vulgaris cells (CCAP 211/11B) from Algae and Protozoa, SAMS Research Services Ltd, Dunstaffnage Marine Laboratory, UK, were grown in Erlenmeyer flasks, capped with foam stoppers (Fisher Scientific) containing Bold's medium ($NaNO_3$ 25 mg L^{-1}, $CaCl_2.2H_2O$ 2.5 mg L^{-1}, $MgSO_4.7H_2O$ 7.5 mg L^{-1}, $K_2HPO_4.3H_2O$ 7.5 mg L^{-1}, KH_2PO_4 17.5 mg L^{-1}, NaCl 2.5 mg L^{-1}, $FeCl_3.6H_2O$ 0.058 mg L^{-1}, $MnCl_2.4H_2O$ 0.024 mg L^{-1}, $ZnCl_2$ 0.003 mg L^{-1}, $CoCl_2.6H_2O$ 0.001 mg L^{-1}, $Na_2MoO_4.2H_2O$ 0.0024 mg L^{-1}) without EDTA[16] under continuous illumination at 2,646 lux (n = 33, SE = 154) and 25 ± 0.5 ºC (LMS Cooled Incubator, Model 300WA). Cells were shaken by hand once a day to avoid sedimentation. All nutrients were reagent grade.

Cells were cultured for 3-5 days in Bold's media free of arsenic and supplemented with 0.5% w/v dextrose (Oxoid, bacteriological grade). Cells were then exposed to As(III) (sodium arsenite), DMA (dimethylarsinic acid) and As(V) (sodium arsenate) in different and independent experiments.

For total extraction (acid microwave-assisted extraction), cells were transferred to Teflon tubes, 4 mL of concentrated nitric acid were added and the mixture was digested in a MARS XPRESS microwave digestion system (Method EPA 3051A 2007).

For the sonication extraction, cells were transferred to 15 mL centrifuge tubes; 2 mL of 1% formic acid was added and the mixture was sonicated for 30 s using a Minidelta 8935 generator (FFR ultrasonics, 500W, 35kHz) fitted with a 3 mm titanium micro-tip. The micro-tip was rinsed with methanol then sonicated in 1% formic acid for 10 s between each extraction to avoid cross-contamination.

Total arsenic was quantified using an ICP-MS (X series II, Thermo Scientific) in CCT mode with He/H as collision cell gas using 20 µg L^{-1} gallium as internal standard. The instrument was tuned daily using a 10 µg L^{-1} In, Ce, Co, U, Li solution. The certified reference material SRM 2669 (National Institute of Standards and Technology, total and arsenic species in human frozen urine) was used for quality control.

Extraction: comparative analysis

The extraction protocol using focused sonication was compared with extraction by grinding in liquid nitrogen[3,4,5,6] as well as with total extraction using microwave-assisted acid digestion of *C. vulgaris* cells exposed to 100 mg L^{-1} As(V) for 5 days and of commercially available Kelp Powder (Galloway's, Richmond, BC, Canada), which has been used for determination of arsenosugars (total arsenic: 27.7 ± 0.08 µg g^{-1} [10,17,18]. Kelp powder was dried and 0.3 g was taken for analysis. *C. vulgaris* cells were harvested by centrifugation (3,000 *g*, 6 min, Rotina 420R), washed three times with deionised water and immediately freeze-dried (Scanvac Coolsafe 55-4). Approximately 0.02 g of dried cells was taken and extraction was carried out.

Extraction of As-GS/PC complexes

After arsenic exposure, cells were washed with deionised water and harvested at 4 ºC. Cells were extracted by addition of 2 mL, 1% formic acid, and focused sonication was applied for 30 s in an ice bath to minimise the effect of increase in local temperature. The resulting extracts were centrifuged further to 16,000 *g* for 3 min at 4 ºC (Eppendorf 5415R) and filtered through 0.45 µm syringe filters (Supelco). This method was compared with the established formic acid extraction at -80°C[5].

Cells were harvested and treated in the same way with As(III), then the two extraction methods were performed (grinding in liquid nitrogen vs. focused sonication) and samples were analysed with the same HPLC-ICPMS/ES-MS method.

HPLC-ICPMS/ES-MS method for arsenic speciation

The analysis was performed immediately after extraction by using HPLC online with high resolution HR-ICPMS and ES-MS/MS. Separation was achieved in a Discovery C_{18} (15 x 2.1 mm, 5 µm) column. A gradient elution was used with 0.1% formic acid (eluent A) and 99.9% HPLC grade methanol (eluent B). The detailed elution profile was as follows: 0-20 min linear increase 0-20% B, 20-30 min 20%B, 30-32 min 20-0% B and 32-40 min 0%B. The flow rate was 0.75 mL min^{-1} [8]. The injection volume was 75 µL. The flow from the HPLC was divided ¼ parts to the HR-ICP-MS and ¾ to the ES-MS/MS. The oven temperature was 30 ºC and auto sampler temperature 4 ºC.

The HR-ICP-MS (Element II, Thermo Scientific) was tuned daily and post column make up flow was achieved with a tee connector and 20 µg L^{-1} gallium in 1% HNO_3 as internal standard; the monitored masses were: m/z 75 (As), m/z 69 (Ga) and m/z 34 (S) in medium resolution. The ES-MS/MS (Orbitrap Discovery LTQ-XL, Thermo Scientific) was a set up with spray voltage 4.5 V, capillary temperature 320 ºC and tube lens 120 V. The ES-MS/MS was set up in positive scan mode from m/z 50 to 2000. The results for the exact mass were obtained from the chromatograms in the ES-MS/MS analysis in scan mode after a high resolution calibration and accurate mass calibration were performed following the manufacturer's instructions (Thermo Scientific).

Results

Extraction: comparative analysis

The focused sonication method was able to achieve 94.7% and 71.1% recovery in Kelp powder and *C. vulgaris* cells respectively compared with acid microwave-assisted extraction. The results for comparisons can be observed in Table 1.

Table 1 Comparison of total (acid microwave-assisted digestion) and focused sonication extraction (30 s, 500 W, 35 kHz) in *C. vulgaris,* commercially available Kelp powder and SRM 2669

Sample	*µgAs/g*		*% recovery*
Total extraction C. vulgaris	83.2	SE[a] = 2.04, n[b] =6	
Sonication C. vulgaris	59.2	SE = 1.14. n = 6	71.1 %
Total extraction Kelp	23.5	SE = 0.60, n = 9	
Sonication Kelp	22.2	SE = 0.28, n = 11	94.7 %
Quality control	*µgAs/L*		
SRM 2669	48.1	% RSD[c] 2.16	
Certified value	50.7	± 6.3 (95 % CI)	

[a] Standard Error
[b] Number of samples
[c] Relative Standard Deviation

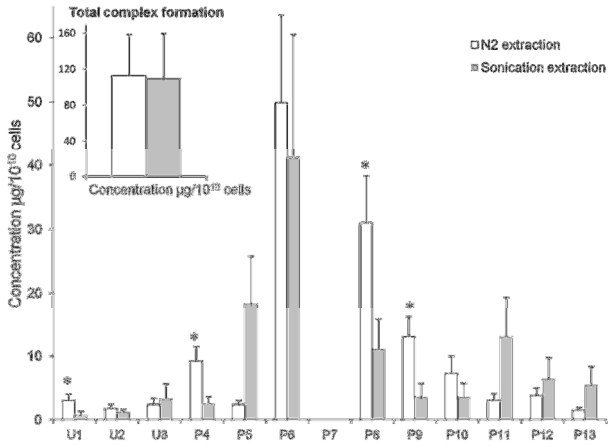

Fig.1 Comparative arsenic speciation analysis, extraction by grinding with N_2 and focused sonication (30 s, 500 W, 35 kHz) in *C. vulgaris* cells exposed to 50 mg L^{-1}As(III) for 48 h, inset total complex formation, vertical bars indicate +1 SE, n=5, * denotes statistical difference (two-sample t-test, p<= 0.05), † U indicates unknown, P peptides identified and the number corresponds to peaks in Fig S3

Extraction of As-GS/PC complexes: qualitative analysis

Cells exposed to As(III) were found to form complexes of arsenic bound to GS/PC. Signals for GSH, GSSG, oxidised PC_2, reduced PC_2, oxidised PC_3, reduced PC_3, γ-(Glu-Cys), γ-(Glu-Cys)-Ala, γ-(Glu-Cys)$_2$, γ-(Glu-Cys)$_2$-Ala and γ-(Glu-Cys)$_3$-Ala as well as their respective arsenic bound complexes were observed. Because of their complexity, the ES-MS and ICP-MS chromatograms are shown in two separate graphs: unbound and bound peptides, Fig 2 and 3 respectively.

The difference between the experimental and exact mass did not exceed 2.2 ppm for any of the As-GS/PC complexes (Table 2).

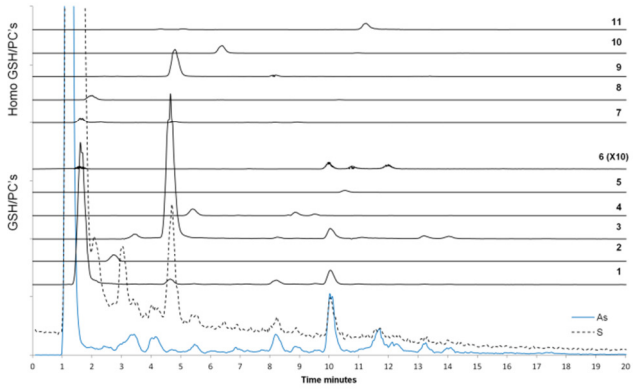

Fig.2 Unbound GSH/PC and homo-GSH/PC, ES-MS and HR-ICP-MS chromatograms of C. vulgaris cells exposed to 50 mg L^{-1} As(III) for 48 h, extracted in 1% formic acid 30 s focused sonication. Peak identification (m/z) (scale) (retention time): **1** GSH (308) (1) (1.61), **2** GSSG (613) (1) (2.75), **3** Reduced PC_2 (540) (1) (4.65), **4** Oxidised PC_2 (538) (1) (5.42), **5** Reduced PC_3 (772) (1) (10.58), **6** Oxidised PC_3 (770) (10) (9.99/12.04), **7** γ-(Glu-Cys) (251) (1) (1.61), **8** γ-(Glu-Cys)-Ala (322) (1) (2.01), **9** γ-(Glu-Cys)$_2$ (483) (1) (4.80), **10** γ-(Glu-Cys)$_2$-Ala (554) (1) (6.40), **11** γ-(Glu-Cys)$_3$-Ala (715) (1) (11.25), As and S signals from HR-ICP-MS

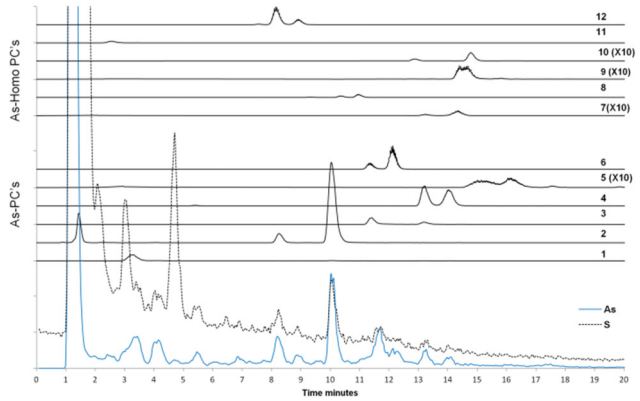

Fig.3 Bound GS/PC and homo-GS/PC, ES-MS and HR-ICP-MS chromatograms of C. vulgaris cells exposed to 50 mg L^{-1} As(III) for 48 h, extracted in 1% formic acid 30 s focused sonication. Peak identification (m/z) (scale) (retention time): **1** As(III)-PC_2 (630) (3.29), **2** GS-As(III)-PC_2 (460) (1) (8.31/10.07), **3** As(III)-PC_3 (844) (1) (11.42/13.23), **4** As(III)-$(PC_2)_2$ (576) (1) (13.23/14.12), **5** As(III)-PC_4 (1076) (1) (15.18/16.22), **6** MMA(III)-PC_2 (628) (1) (11.42/12.17), **7** As(III)-(Glu-Cys)$_3$-Ala (858) (10) (14.35), **8** GS-As(III)-(Glu-Cys)$_2$-Ala (467) (1) (10.98), **9** As(III)-((Glu-Cys)$_2$)$_2$-Ala (583) (10) (14.39), **10** MMA(III)-(Glu-Cys)$_2$-Ala (642) (10) (14.78), **11** As(III)-(Glu-Cys)$_2$ (573) (1) (2.59), **12** GS-As(III)-(Glu-Cys)$_2$ (431) (1) (8.14/8.89), As and S signals from HR-ICP-MS

Cells exposed to DMA also form one complex of arsenic bound to GS/PC. Only signals for GSH, reduced PC_2, Oxidised PC_2 and $DMAS^V$-GS were observed. The ES-MS and ICP-MS chromatograms are shown in Fig 4. Reduced PC_2 co-eluted with $DMAS^V$-GS and gave signals for S and As, however the signal for arsenic can only come from $DMAS^V$-GS since PC_2 is not complexed with arsenic.

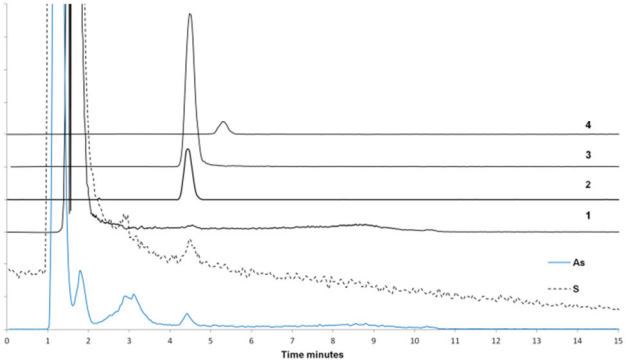

Fig.4 GSH/PC and bound As-GS/PC, ES-MS and HR-ICP-MS chromatograms of *C. vulgaris* cells exposed to 50 mg L^{-1} DMA for 48 h, extracted in 1% formic acid 30 s focused sonication. Peak identification (m/z) (scale) (retention time): **1** GSH (308) (1) (1.58), **2** $DMAS^V$-GS (444) (1) (4.48), **3** Reduced PC_2 (540) (1) (4.52) and **4** Oxidised PC_2 (538) (1) (5.28), As and S signals from HR-ICP-MS

In contrast *C. vulgaris* cells exposed to As(V) did not produce any signal for As bound to GS/PC at any of the concentrations used (1-200 mg L^{-1}) or the exposure times (1, 4, 24 and 48 h) † (Fig S1 and S2).

Table 2 Molecule identification and mass difference for GS/PC complexes found in *C. vulgaris* cells exposed to 50 mg L^{-1} As(III) and DMA analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL

Molecule	Formula	Monoisotopic mass ($M+H^+$ or $M+2H^+$)	Experimental mass	Difference ppm
GSH/PC				
GSH	$C_{10}H_{17}N_3O_6S$	308.0916	308.0912	-1.34
GSSG	$C_{20}H_{32}N_6O_{12}S_2$	613.1598	613.1598	0.00
Reduced PC_2	$C_{18}H_{29}N_5O10S_2$	540.1434	540.1433	-0.16
Oxidised PC_2	$C_{18}H_{27}N_5O_{10}S_2$	538.1278	538.1289	2.04
Reduced PC_3	$C_{26}H_{41}N_7O_{14}S_3$	772.1952	772.1952	-0.01
Oxidised PC_3	$C^{26}H_{39}N_7O_{14}S_3$	770.1795	770.1795	0.00
Reduced PC_4	$C_{34}H_{53}N_9O_{18}S_4$	1004.2470	Not found	
As(III)-PC_2	$C_{18}H_{28}N_5O_{11}S_2As$	630.0443	630.0437	-0.88
As(III)-PC_3	$C_{26}H_{38}N_7O_{14}S_3As$	844.0933	844.0931	-0.19
GS-As(III)-PC_2	$C_{28}H_{43}N_8O_{16}S_3As$	460.0666	460.0663	-0.06
		919.1253	919.1247	-0.69
As(III)-$(PC_2)_2$	$C_{36}H_{54}N_{10}O_{20}S_4As$	576.0925	576.0923	-0.37
As(III)-PC_4	$C_{34}H_{50}N_9O_{18}S_4As$	1076.1451	1076.1455	0.37
MMA(III)-PC_2	$C_{19}H_{30}N_5O_{10}S_2As$	628.0728	628.0729	0.12
$DMAS^V$-GS	$C_{12}H_{23}N_3O_6S_2As$	444.0244	444.0247	0.56
Ala GSH/PC				
γ-(Glu-Cys)-Ala	$C_{11}H_{19}N_3O_6S$	322.1073	322.1072	-0.21
γ-(Glu-Cys)$_2$Ala	$C_{19}H_{31}N_5O_{10}S_2$	554.1591	554.1578	-2.20
As(III)-γ-(Glu-Cys)$_3$-Ala	$C_{27}H_{40}N_7O_{14}S_3As$	858.1090	858.1082	-0.87
GS-As(III)-γ-(Glu-Cys)$_2$-Ala	$C_{29}H_{45}N_8O_{16}S_3As$	467.0744	467.0744	0.09
As(III)-γ-((Glu-Cys)$_2$)$_2$-Ala	$C_{37}H_{57}N_{10}O_{20}S_4As$	583.1003	583.1010	1.19
MMA(III)-γ-(Glu-Cys)$_2$-Ala	$C_{20}H_{32}N_5O_{10}S_2As$	642.0885	642.0889	0.66
desGly GSH/PC				
γ-(Glu-Cys)	$C_8H_{14}N_2O_5S$	251.0702	251.0706	1.55
γ-(Glu-Cys)$_2$	$C_{16}H_{26}N_4O_9S_2$	483.1219	483.1217	-0.58
γ-(Glu-Cys)$_3$	$C_{24}H_{38}N_6O_{13}S_3$	715.1737	715.1747	1.41
As(III)-γ-(Glu-Cys)$_2$	$C_{16}H_{25}N_4O_{10}S_2As$	573.0306	573.0318	2.11
GS-As(III)-γ-(Glu-Cys)$_2$	$C_{26}H_{40}N_7O_{15}S_3As$	431.5558	431.5558	-0.17

When cells were exposed to 50 mg L^{-1} of As(III), As(V) and DMA, total arsenic and total As-GS/PC complex formation were calculated in independent experiments. Total arsenic was 269.8 µg /10^{10} cells, 24.3 µg /10^{10} cells and 90.0 µg /10^{10} cells for cells exposed to As(III), As(V) and DMA respectively. The amount of As-GS/PC complex formed was 36%, 0% and 0.8% for As(III), As(V) and DMA respectively.

The difference between the experimental and exact mass for the molecules found in cells exposed to DMA did not exceed 1.86 ppm for any of the As-GS/PC complexes.

The signals for GS-As(III)-PC_2 (m/z 460 and 919) MMA(III)-PC_2, As(III)-PC_3, As(III)-$(PC_2)_2$, As(III)-PC_4, $DMAS^V$-GS, γ-(Glu-Cys)$_2$Ala (m/z 554), As(III)- γ-(Glu-Cys)$_3$-Ala (m/z 858), As(III)- γ-((Glu-Cys)$_2$)$_2$-Ala (m/z 583), MMA(III)-γ-(Glu-Cys)$_2$-Ala (m/z 642), γ-(Glu-Cys)$_2$ (m/z 483) and GS-As(III)-γ-(Glu-Cys)$_2$ (m/z 431) were strong enough to isolate and perform

MS/MS analysis. †(The complete dissociation pathways are shown in Fig S4-S16 and Table S1). The fragments found and the fragmentation pathways agree with previously published data[4,5,8,19].

Focused sonication was compared with extraction by grinding using liquid nitrogen. The results for quantification of arsenic are shown in Fig 1. The results show that there is comparable total complex extraction. However, individual peak stability was favoured for peaks 5 (As(III)-γ-(Glu-Cys)$_2$), 11 (As(III)-(PC$_2$)$_2$ / As(III)- γ-(Glu-Cys)$_3$-Ala / As(III)- γ-((Glu-Cys)$_2$)$_2$-Ala / MMA(III)-γ-(Glu-Cys)$_2$-Ala, 12 and 13 (As(III)-PC$_4$) for samples extracted using focused sonication, whereas integrity was better for peaks 4, 6, 8, 9 and 10 for the conventional extraction method.

Discussion

Extraction efficiency using focused sonication in *C. vulgaris* cells was lower than that of Kelp powder. One reason could be that *C. vulgaris* cells produce more lipids (up to 26% by weight[20]) and these could combine with arsenic to form fat-soluble arsenic compounds[21]. Previous studies found extraction efficiencies in *C. vulgaris* cells in the range of 11-27%[9]. Focused sonication has been used to extract arsenic from environmental samples but without giving specific information in the conditions[22,23]. In this study, a considerable improved extraction efficiency of 71.1% was achieved. It appears that focused sonication funnels more energy enabling more targeted intermolecular force disruption and thus mobilising more arsenic into aqueous solution. The method therefore achieves representativeness of water soluble arsenic species in *C. vulgaris* with the potential to achieve species integrity by minimisation of the treatment time (30 s).

The method shows that there is no indication of any of the complexes suffering disintegration caused by the high energy sonication. Moreover, we report the presence of oxidised PC$_2$, reduced PC$_2$, oxidised PC$_3$ and reduced PC$_3$ in *C. vulgaris* cells challenged with arsenic. These peptides have only been reported in *C. vulgaris* in experiments using oxygen-free extraction with cells challenged with cadmium and selenate[24,25]. It has also been reported that 90% of these peptides can be lost by freeze-drying alone[5]; hence their presence in this study notably denotes stability of the extraction.

Arsenic was complexed with GS/PC and homo-GS/PC only when exposed to As(III) inside *C. vulgaris* cells. We report the presence of GS-As(III)-PC$_2$, As(III)-(PC$_2$)$_2$, MMA(III)-PC$_2$, As(III)-PC$_3$ and As(III)-PC$_4$ in *C. vulgaris* cells which have not been previously documented. Its presence has been previously reported only in *Holcus lanatus*, *Pteris cretica* and *Helianthus annuus*[13,26,27]. We also report the presence of DMAS^V-GS in *C. vulgaris* cells. The production of this complex *in vivo* has only been reported once in *Brassica oleracea* plants[4].

To our knowledge, the presence of intact As-homo-GS/PC complexes has not been previously reported in any organism. Thus we report for the first time As bound to homo-GS/PC in the following molecules: As(III)- γ-(Glu-Cys)$_3$-Ala, GS-As(III)- γ-(Glu-Cys)$_2$-Ala, As(III)- γ-((Glu-Cys)$_2$)$_2$-Ala, MMA(III)-γ-(Glu-Cys)$_2$-Ala, As(III)-γ-(Glu-Cys)$_2$ and GS-As(III)-γ-(Glu-Cys)$_2$.

It has been demonstrated that desGly-PCs (where Gly is absent in GS) are not generated as immediate by-products of PC synthase action when PCs are formed[28]. Moreover, it has also been demonstrated the presence of desGly-GSH or Ala-GSH alone is sufficient for the formation of homo-PCs. Thus, PC synthase can catalyse the synthesis of homo-PCs without the need for a specific enzyme[29]. Therefore, the sole presence and production of desGly-GSH and Ala-GSH in *C. vulgaris* cells before production of homo-PCs is very important in the first instance. In this study unbound GSH/PCs were not quantified due to the high detection limits of sulphur using single quadrupole ICP-MS. However, the fact that there is no reported production of As-homo-GS/PC complexes in the literature and the fact that these complexes can form without the need for a specific enzyme gives further evidence that the method used in the present study for extraction of As-GS/PC complexes promotes complex stability, otherwise other studies would have already reported the formation of As-homo-GS/PC that form parallel to As-GS/PC.

We have calculated accurate masses (< 2.2 ppm) and MS/MS spectra for all As-GS/PC and 4 of the 6 As-homo-GS/PC complexes. This along with the elemental analysis provides extremely strong evidence of their presence. These findings suggest that *C. vulgaris* cells possess not only the mechanism for GS/PC complexation but also the specific transport system to store these complexes in the vacuole (with conditions suitable for stability such as acidic pH)[30].

Several of the arsenic bound complexes have two peaks (probably corresponding to different isoforms) and some molecules elute together (with the specific conditions used in the present study). These two facts may have hampered the identification in previous studies of the molecules mentioned above. Nevertheless this does not affect the quantification using ICP-MS due to the fact that all the signals observed after 7 min correspond to a phytochelatin related molecule bound to arsenic. There are however, two molecules that are not easy to quantify due to their early elution from the column: As(III)-PC$_2$ and As(III)-(Glu-Cys)$_2$.

Arsenic was not complexed with GS or PC in the presence of As(V). One possible reason is that As(V) is hardly reduced by this alga (only 1-6%)[9] and the affinity of sulphur for As(III) is much stronger than for As(V). Therefore hard As(V) will only bind to biothiols such as GS after thiolation and two methyl group has rendered it softer[4]. Many studies have described the induction of PCs upon exposure to As(V), in this study we give evidence that the formation of PCs only responds to As(III) and the formation of PCs observed in other studies may had been due to previous reduction of As(V) rather than direct induction of PC formation[2,31,32].

C. vulgaris cells are able to detoxify As(III), As(V) and DMA since it thrives in large arsenic concentration. Detoxification of As(III) and to a lesser extent DMA appears to rely on GS/PC complexation. In contrast a different mechanism is likely to be involved in As(V) detoxification. Some authors have reported the induction of GSH and PC by the presence of As(V) e.g. *C. vulgaris*[1] and *Chlamydomonas reinhardtii*[2], but this may have been the effect of arsenate being reduced and subsequently complexed with GS/PC and therefore erroneously interpreted[1,2].

These findings also open the possibility to study the arsenic detoxification mechanism in plants using what can be considered a model organism: *C. vulgaris* cells. This is because the algae appears to not be able to reduce As(V), the mechanism of As(III)

and As(V) detoxification can be studied in a mutually exclusive manner thus reducing erroneous interpretations.

Conclusion

The method reported here represents an alternative to the conventional extraction method (grinding with liquid nitrogen) which is both rapid, reducing the extraction time significantly from 1 h to 30 s and less sample is lost in each extraction. However, fibrous rich plant samples could be difficult to extract with this method. In addition, because dry weight is difficult to determine using sonication (where the sample remains wet), another surrogate to the amount of sample is needed in order to get accurate quantification of the molecules. This can be done by measuring chlorophyll a (by fluorescence or absorbance) and relate the measurement to cell numbers and thus reporting the amount of arsenic found per number of cells.

Integrity of at least eight peptides (oxidised PC_2, reduced PC_2, oxidised PC_3, reduced PC_3, γ-(Glu-Cys), γ-(Glu-Cys)-Ala, γ-(Glu-Cys)$_2$, γ-(Glu-Cys)$_2$-Ala and γ-(Glu-Cys)$_3$-Ala) as well as twelve As-GS/PC complexes (GS-As(III)-PC_2, As(III)-$(PC_2)_2$, MMA(III)-PC_2, As(III)-PC_3, As(III)-PC_4, $DMAS^V$-GS, As(III)-(Glu-Cys)$_3$-Ala, GS-As(III)-(Glu-Cys)$_2$-Ala, As(III)-((Glu-Cys)$_2$)$_2$-Ala, MMA(III)-(Glu-Cys)$_2$-Ala, As(III)-(Glu-Cys)$_2$ and GS-As(III)-(Glu-Cys)$_2$) was ensured. This has never been achieved before in microalgae samples. Representativeness of the water soluble species inside these cells was achieved with 71.1% extraction efficiency.

Improved analytical approaches of the kind described in this paper represent an important contribution to the development of a deeper understanding of the processes taking place when inorganic arsenic challenges algal cells. *C. vulgaris* has an established potential as an organism for detoxification of arsenic contaminated drinking water, a major global public health issue and as such, the findings may also warrant potential impact by contributing to future improved/optimised arsenic remediation techniques.

Notes and references

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† Electronic Supplementary Information (ES) available: [Methods, As(V) exposure experiments, MS/MS information].

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